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Distribution of developmentally regulated trans-sialidases in the Kinetoplastida and characterization of a shed trans-sialidase activity from procyclic *Trypanosoma congolense*

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Abstract

The expression of developmentally regulated sialidase and trans-sialidase activities in kinetoplastid protozoa was investigated. The occurrence of these enzymes was found not to be a common feature among the *Kinetoplastida*, but to be restricted to distinct developmental life cycle stages of only a few species. While sialidases without trans-sialylating activities were demonstrated in *Trypanosoma vivax* and *T. rangeli*, trans-sialidase activity is expressed throughout the *brucei*-group and in *T. congolense*. Neither *T. evansi*, nor *T. equiperdum* express sialidases or trans-sialidases. Furthermore, the absence of both, sialidase and trans-sialidase activities was proven in the *Leishmania*, *Crithidia*, *Herpetomonas*, *Leptomonas* and *Phytomonas*, respectively. In all species tested, the occurrence of sialic acids coincides with the expression of trans-sialidase activity. Those parasites, which lack trans-sialidases or only display regular sialidases, also lack cell-bound sialic acids. The regular sialidase activity from bloodstream form *T. vivax* was characterized. The trans-sialidase from *T. congolense* is restricted to the procyclic culture forms and is shed into the culture medium. The enzyme has a pH-optimum at pH 7.0, displays sensitivity towards chlorides and is resistant against commonly used sialidase inhibitors. *T. congolense* trans-sialidase transfers preferentially α(2–3)-linked sialic acids onto terminal β-galactose residues. Also hydroxylated sialic acids (Neu5Gc) are transferred. The major glycoprotein GARP from procyclic *T. congolense* was identified as one potential natural sialic acid acceptor on the parasite's surface. In order to facilitate the characterization of trans-sialidases a novel, fluorimetric trans-sialidase assay was developed.

Key words: Trans-sialidase; Sialidase; *Kinetoplastida*; *Trypanosoma congolense*; *Trypanosoma vivax*

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Abbreviations: CMP, cytidine-5'-monophosphate; GARP, glutamate and alanine-rich protein; GPI, glycosylphosphatidylinositol; MU-Neu5Ac, 4-methylumbelliferyl-α-D-N-acetylneuraminic acid; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycoloylneuraminic acid; Neu5Ac2en, 2-deoxy-2,3-didehydro-N-acetyl neuraminic acid; PARP, procyclic acidic repetitive protein; PMSF, phenylmethylsulfonyl fluoride; TLCK N-tosyl-L-lysine chloromethyl ketone.

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Introduction

The *Kinetoplastida* represent a prominent order of flagellated protozoa containing several species threatening man and his domestic animals, especially among the genera *Trypanosoma* and *Leishmania*. The life cycle of several African trypanosomes is punctuated by differentiation events triggered by the dramatic environmental changes that accompany cyclical transmission by an insect vector, the tsetse fly (*Glossina spec.*) (Vickerman et al., 1988). Trypanosomes are ingested with the tsetse fly's bloodmeal and transferred back to the mammal with the saliva. In the fly's midgut, the bloodstream form trypanosomes differentiate into procyclic insect stages, resulting in basic changes of the biosynthetic apparatus, which is accompanied by the loss of the protective VSG coat and the parallel acquisition of a similar amount of invariant procyclic acidic repetitive proteins (PARP=procyclin) (Roditi and Pearson, 1990). These procyclic surface glycoproteins are anchored to the membrane via a modified glycosylphosphatidyl-inositol (GPI) structure (Ferguson et al. 1993). Although tsetse-transmission is a feature of many African trypanosome species, it is not generally required. *Trypanosoma vivax* for example has the capability of using vectors other than *Glossina*-species and *T. equiperdum* and *T. evansi* omit a life cycle stage within the fly completely (Hoare, 1972).

For more than two decades, African trypanosomes have attracted great scientific interest. A variety of biochemical and molecular biological phenomena are either unique to these parasites or have been described here for the first time (Hajduk et al., 1992). Trans-sialylation is probably the most recent example.

Sialic acids are a group of more than 40 modifications of the nine-carbon-sugar neuraminic acid. Due to their acidic nature and high variability, sialic acids, which usually reside in terminal positions on oligosaccharide chains of glycoproteins and glycolipids, have a marked influence on a multitude of cellular recognition and masking processes (Schauer, 1982; Schauer, 1985; Varki, 1992). As key enzymes of sialic acid catabolism sialidases (EC 3.2.1.18) are also involved in these biological and biochemical events (Corfield et al., 1991; Corfield, 1992). Trypanosomal sialidases have been reported from *T. cruzi* (Pereira, 1983), *T. rangeli* (Reuter et al., 1987) and, more recently, from *T. brucei* (Engstler et al., 1992b). Both, the *T. cruzi* and *T. brucei* enzymes act as unique trans-sialidases which are not only capable of cleaving terminally bound sialic acids but also transfer these sugars onto new glycosidic linkages (Zingales et al., 1987; Schenkman et al., 1991; Engstler et al., 1992a). There is strong evidence that this mechanism may be critically involved in cell invasion by *T. cruzi* and survival of *T. brucei* within the tsetse fly (Schenkman and Eichinger, 1993; Engstler and Schauer, 1993). In both cases trans-sialidases enable the parasites to transfer sialic acids from the environment (e.g. from serum glycoproteins or erythrocytes) onto trypanosomal surface molecules. In *T. brucei* the surface-located trans-sialidase sialylates the major surface protein (PARP= procyclin) of the procyclic form (Engstler et al., 1993; Pontes De Carvalho et al., 1993). In this way a negatively charged glycocalyx is formed, which is thought to protect *T. brucei* against the vector's digestive system.

Here we show that trans-sialylation is not a common feature among kinetoplastid protozoa, but is restricted to some species and developmental stages. Thus, we conclude that the expression of trans-sialidase may be bound up with major biological advantages for the parasite.

Materials and methods

Parasites: Procyclic culture forms of *T.b. brucei* and *T.b. rhodesiense* were grown in medium SDM-79 (Brun and Schönenberger, 1979) or medium HX25M (Cross and Manning, 1973), respectively, and the corresponding mammalian life cycle stages in mice. *T.b. gambiense* (bloodstream forms) were grown in immunosuppressed *Mastomys natalensis*.

T. evansi, *T. equiperdum*, *T. congolense*, *T. vivax* and *T. rangeli* bloodstream forms were grown in mice. *T. congolense* procyclics were cultivated in SM/SDM 79 (Kaminsky et al., 1988). Vector stages of *T. rangeli*, *T. cruzi*, *Crithidia* and *Herpetomonas* were grown in Mattei medium (Mattei et al., 1977). Mammalian stage *T. cruzi* cells were produced in WI38 human fibroblasts grown in MEM with 10% FBS. *Entamoeba histolytica* was produced in TYI-S-33 (Diamond et al., 1978) and *Giardia lamblia* (human-derived) in a modified form of this culture medium (Keister, 1983).

Materials: All chemicals used in the present study were of analytical grade. Q-Sepharose Fast Flow was from Pharmacia (Freiburg, FRG). [D-Glucose-1-^{14}C]lactose (60 mCi/mmol) was from Amersham (Braunschweig, FRG). 4-Methylumbelliferyl-β-D-galactopyranoside (Gal-MU) was purchased from Sigma (Deisenhofen) and stored in DMSO. 4-Methylumbelliferyl-α-D-N-acetylneuraminic acid (MU-Neu5Ac) was from Dr. Pallmann GmbH (München, FRG). All other compounds used here as putative sialic acid donors were prepared as described elsewhere (Engstler et al., 1992b). PARP was isolated from freshly harvested procyclic *T.b. brucei* STIB 247 according to Ferguson et al. (1993). Enriched *T. congolense*-GARP was a gift from Dr. David Jefferies (Glasgow, Scotland). Gangliosides were isolated from horse erythrocytes and subsequently Neu5Gc-α(2–3)lactose was prepared as described earlier (Engstler et al., 1993). Oligosaccharides used as potential trans-sialidase acceptor compounds were a gift from Dr. J.-C. Michalski (Lille, France). N-(4-Nitrophenyl)oxamic acid was donated by Dr. Andrea Vasella (Zürich, Switzerland) and Siastatin B was a kind gift of Dr. Nishimura (Tokio, Japan). Trasylol was obtained from Bayer (Leverkusen, FRG).

Enzymes: Triton CF-54 extracts from procyclic *T.b. brucei* or purified *T.b. brucei* trans-sialidase were used as a source for *T.b. brucei* enzyme (Engstler et al., 1993) and culture supernatants from epimastigote *T. rangeli* and procyclic *T. congolense*, respectively, were used as a source for the corresponding enzymes. Freshly harvested parasites and culture supernatants from other kinetoplastid and related species were tested for both, trans-sialidase and sialidase activity. Sialidase from *Vibrio cholerae* was purchased from Behringwerke (Marburg, FRG).

Assays: Unless otherwise stated enzyme incubations were performed at 37°C. Controls of enzymatic activities were performed with heat-denatured samples. All values are means (±standard deviation) of at least 3 independent experiments. Enzyme inhibition studies were performed in 5 independent experiments with overall 60 single point measurements at various inhibitor concentrations. All reactions were carried out in the linear range of enzyme activities. In the conventional assay (Schenkman et al., 1991), trans-sialidase activity was measured in a final volume of 50 to 100 µl in 50 mM Bis-Tris buffer, pH 6.9, containing 0.4% Triton CF-54 and proteinase inhibitors (1 mM PMSF/0.1 mM EDTA/0.1 mM TLCK/5 µg ml^{-1} pepstatin/5 µg ml^{-1} leupeptin/5 µg ml^{-1} Trasylol (buffer A)). Routinely, 1 mM Neu5Ac-α(2–3)-lactose was used as sialic acid donor and 1 mM lactose containing

200 000 dpm D-[^{14}C]-lactose as acceptor. After 10–60 min at 37°C, 1 ml water was added followed by passage through 1 ml Q-Sepharose Fast Flow equilibrated and run in water. After washing the column with 5 ml water, bound activity was eluted with 1 ml 1 M ammonium formate. This radioactive assay was used for trans-sialidase activity screening.

In a novel fluorimetric trans-sialidase assay 4-methylumbelliferyl-β-D-galactopyranoside (Gal-MU) was used as sialic acid acceptor. 2 mM Gal-MU was incubated with trans-sialidase and 1 mM Neu5Ac-α(2–3)-lactose as sialic acid donor in a final volume of 50 to 100 µl in buffer A. After 10–60 min at 37°C, 8 vol. water and 1 vol. Q-Sepharose were added. Following resupension, the reaction mixture was centrifuged for 2 s and the resulting Q-Sepharose pellet was washed 3-times with 9 vol. water. The anion exchange resin was resuspended in 2 vol. 1N HCl and incubated for 20 min at 96°C. Following centrifugation, the supernatant was adjusted to pH 10 and fluorimetric reading was done directly (excitation 365 nm, emission 450 nm). This non-radioactive test was used for all trans-sialidase characterization studies presented here.

Sialidase activity was routinely tested with MU-Neu5Ac, 0.1 mM final concentration as described earlier (Engstler et al., 1992b). When fluorimetric reading was done using a microtiter fluorimeter (Cytofluor 2300, Millipore) and CoStar 96 well microtiter plates, enzyme reaction volume was 100 µl and the reaction was terminated by the addition of 200 µl of double concentrated glycine buffer (20 g/l glycine, 7 g/l NaCl, 8.8 g/l Na_2CO_3), pH 10. The measurement of fluorescence was calibrated using methylumbelliferone standards with each experiment.

For determination of pH or temperature optima, enzymes were incubated in appropriate buffer systems with various pH values, or in buffer A at different temperatures.

The sialic acid content of parasite cells was determined from lyophilized cells by a fluorimentric HPLC procedure (Hara et al., 1989) as described by Engstler et al. (1993). Briefly, up to 5×10^8 cells were washed extensively on ice and the resulting cell pellet was resuspended in 50–100 µl of 0.1 M HCl. Following acid hydrolysis for 60 min at 80°C and centrifugation, 10–20 µl of the supernatant was derivatized with 1,2-diamino-4,5-methylenedioxybenzole and analyzed by fluorimetric HPLC using a RP-18 cartridge (25 × 0.4 cm; Merck, Darmstadt, FRG). The procedure was calibrated with standard sialic acids.

In order to determine the cellular localization of *T. vivax* sialidase, isolation of plasma membranes and protease treatment of intact cells were carried out as described by Engstler at al. (1992b).

Results and Discussion

Occurrence of sialidases and trans-sialidases

Research so far suggests that the expression of trans-sialidases is restricted to trypanosomes and most of the work on these unique enzymes has been focused on the bloodstream trypomastigote stage of *Trypanosoma cruzi* (reviewed in Cross and Takle, 1993). In *T. cruzi*, trans-sialylation is involved in interactions between mammalian host cells and the parasites, leading to invasion by the trypanosomes and disruption of the phagosomal membrane (Hall et al., 1992; Schenkman and

Eichinger, 1993). Among the African trypanosomes, *T.b. brucei* is the only species which has been shown to express trans-sialidase activity (Engstler et al. 1992a). This enzyme is developmentally regulated and has been studied in detail (Engstler et al., 1993; Pontes De Carvalho et al., 1993). The aim of the present study was to investigate whether trans-sialylation is a common feature within the *Trypanosomatida*.

Different developmental stages of various strains from several kinetoplastid species were analyzed for the presence of both, sialidase and trans-sialidase activities. The results are summarized in Table 1.

All strains of *T.b. brucei*, *T.b. rhodesiense* and *T.b. gambiense* species displayed trans-sialidase activity within the procyclic stage. However, no enzyme activity was detectable in the corresponding mammalian bloodstream forms. Specific activities and major enzymatic properties of these enzymes are almost identical, e.g. the neutral pH-optima, inhibition behaviour and surface localization.

TABLE 1

Expression of developmentally regulated sialidase and trans-sialidase activities in some kinetoplastid protozoa

Species	Strain	Insect stage		Mammalian stage	
		S	TS	S	TS
Trypanosoma b. brucei	EATRO427	+	+	–	–
	STIB247	+	+	–	–
	STIB345AD	n.d.	n.d.	–	–
	STIB366	+	+	–	–
T.b. rhodesiense	STIB704	+	+	–	–
	STIB364A	+	+	–	–
	STIB809	+	+	–	–
	TH-162/78E(021)	+	+	n.d.	n.d.
T.b. gambiense	TH-17/78E(020)	+	+	–	–
	STIB755	+	+	n.d.	n.d.
T. evansi	STIB779A	0	0	–	–
T. equiperdum	STIB818	0	0	–	–
T. congolense	STIB212	+	+	–	–
T. vivax	IL1392	–	–	+	–
T. rangeli	El Salvador	+	–	–	–
	R1306	+	–	–	–
T. cruzi	Y strain	+	+	+	+
	Esmeraldo	+	+	+	+
Leishmania tropica	MHOM/SU/74/L27	–	–	–	–
L. infantum	LEM768	–	–	–	–
L. brasiliensis	LEM781	–	–	–	–
Crithidia luciliae		–	–	0	0
C. fasciculata		–	–	0	0
Herpetomonas muscarum		–	–	0	0
Phytomonas spec.		–	–	0	0
Leptomonas seymouri		–	–	0	0
Giardia lamblia	G1	0	0	–	–
Entamoeba histolytica	HK9	0	0	–	–

S: sialidase activity; TS: trans-sialidase activity; +: enzyme activity present; –: enzyme activity absent; 0: species lacks life cycle stage; n.d.: not determined.

Interestingly, *T. evansi* and *T. equiperdum*, which are taxonomically closely related to the *brucei*-group, lack trans-sialidase activity. However, these species are being transferred mechanically between their mammalian hosts, thereby omitting the tsetse fly stage. DNA comparisons of *T. evansi* and *T. brucei* (Aratama et al., 1992) have revealed that the genes coding for PARP (=procyclin), which is the major trans-sialylation acceptor on the surface of procyclic *T. brucei*, may be also present in *T. evansi*. Our preliminary data now suggest that also sialidase-like DNA-sequences can be found within the genome of both, *T. equiperdum* and *T. evansi* (M. Engstler, R. Schauer and P. Roggentin, unpublished results). Thus, the expression of active trans-sialidases within the subgenus *Trypanozoon* may correlate with tsetse-transmission.

This assumption is further confirmed by the identification of a trans-sialidase from *T. congolense* (subgenus *Nannomonas*), which is also restricted to procyclic culture forms. Bloodstream trypanosomes (from mouse or from culture) do not reveal enzyme activity. Like the *brucei*-group trypanosomes, *T. congolense* is transmitted by the tsetse fly and undergoes an extensive proliferation stage within the fly's midgut (Vickerman et al., 1988). In agreement with our hypothesis, that trans-sialylation in the tsetse-born stages of African trypanosomes might be essential for these parasites (and therefore is confined to procyclic forms), is the absence of trans-sialidase activity from *T. vivax*. Although *T. vivax* is capable of infecting tsetse flies, the parasites thereby bypass the fly's midgut. Furthermore, *T. vivax* can be transmitted by mechanical means in countries outside the 'tsetse belt' (Gardiner, 1989). Although no trans-sialidase is expressed by *T. vivax*, the bloodstream forms reveal sialidase activity. First reports on the occurrence of a sialidase in *T. vivax* have been published more than a decade ago (Esievo, 1979), however, until now, no information on the enzymatic properties are available. We found the enzyme to be membrane-bound and developmentally restricted to the mammalian life cycle stage. Following purification of plasma membranes by density gradient centrifugation (Engstler et al. 1992b), sialidase activity was exclusively associated with the isolated membranes. Furthermore, the enzyme activity on intact trypanosomes was decreased to 10% within 5 min after the addition of 50 $\mu g\ ml^{-1}$ trypsin. The catalytic optima are at pH 6.0 and 37°C in 100 mM Bis-Tris buffer. The enzymic activity is sensitive towards alkaline pH-conditions (no catalytic activity after 20 min incubation at pH 8.0 in 100 mM TES-buffer). The naturally occurring sialidase inhibitor Neu5Ac2en (Schauer and Corfield, 1981) decreases the catalytic activity to 50% at 0.1 mM concentration, whereas the synthetic sialidase inhibitor N-(4-nitrophenyl) oxamic acid does not affect enzyme activity. Following preincubations in the presence of 1 M NaCl, 82% of the initial sialidase activity was measured. The enzyme activity is increased by the addition of calcium ions (to 122% sialidase activity at 1 mM Ca^{2+}) and is decreased by chelating reagents, such as EDTA (to 27% sialidase activity at 1 mM EDTA). This basic enzyme characteristics of *T. vivax* sialidase significantly differ from those of the trypanosomal trans-sialidases investigated. Especially, the more acidic pH-optimum, the sensitivity towards Neu5Ac2en and the dependence on calcium ions remind on *T. rangeli* sialidase activity (Reuter et al., 1987). However, *T. rangeli* sialidase is shed into the culture medium and its specific activity is more than a hundred times higher, when compared to *T. vivax* sialidase. Thus, the latter two trypanosomal species bear conventional sialidases thereby lacking trans-sialidase activity.

Besides the above trypanosomal species, we also examined members of the genera *Leishmania*, *Leptomonas*, *Herpetomonas* and *Crithidia* for the expression of sialidases or trans-sialidases. Both enzyme activities were absent from all species and strains tested here. Also *Giardia lamblia* and *Entamoeba histolytica*, which have been used as parasitic protozoan not belonging to the order *Kinetoplastida*, lack these enzymes. However, the first trans-sialidase outside the Kinetoplastida has been detected in *Pneumocystis carinii* (M.E.A. Pereira and N. Pavia-Ruz, personal communication).

Occurrence of sialic acids

Both, in *T. cruzi* and *T. brucei*, the expression of trans-sialidases has been shown to correlate with the occurrence of sialic acids on major cell-surface epitopes (Schenkman et al., 1991; Engstler et al., 1993; Pontes De Carvalho et al., 1993). In addition, both species lack the capability to anabolize sialic acids (Schauer et al., 1983; Engstler et al., 1993). Thus, we analyzed a subset of the kinetoplastid strains listed in Table I for the occurrence of cell-associated sialic acids. All strains, which have been shown to express trans-sialidase activity, also revealed cell-bound sialic acids (Table 2). Although *T. congolense* trans-sialidase is shed into the culture medium, sialic acids are present on the surface of the procyclic culture forms. A comparative analysis of the sialic acid contents in trypanosomes of the *brucei*-group and *T. congolense* shows that the latter species possesses about 20 percent more sialic acids when compared to *T. brucei*. This observation is confirmed by the identification of the *T. congolense* surface glycoprotein GARP (Bayne et al., 1993;

TABLE 2

Distribution of cell bound sialic acids in some kinetoplastid protozoa

Species	Amount of sialic acids (ng/10^7 cells)	
	Insect stage	Mammalian stage
T.b. brucei EATRO427[1]	120	2
T.b. brucei EATRO427[2]	0	1
T.b. brucei STIB247	126	n.d.
T.b. rhodesiense STIB704	116	n.d.
T.b. rhodesiense TH-162/78E(021)	90	2
T. evansi 779A	X	1
T. equiperdum STIB818	X	0.5
T. congolense STIB212	146	1.5
T. vivax IL1392	n.d.	2
T. rangeli R1306	2	n.d.
T. cruzi Y strain	n.d.[3]	59
T. cruzi Esmeraldo	n.d.[3]	75
Leishmania tropica MHOM/SU/74/K27	1.5	0.5
L. brasiliensis LEM781	2	0.5
Crithidia luciliae	1	X

All values are means of at least 3 independent experiments. Variations between the measurements were less than 4%. The procedure was calibrated with standard sialic acids (for details see Materials and Methods). All values ≤ 2 ng/10^7 cells are remnants from sializ acids within the culture medium or within the blood samples. X: this species lacks life cycle stage; n.d.: not determined; 1: cells grown in (sialic acid containing) medium SDM79; 2: cells grown in (sialic acid lacking) medium HX25M. 3: sialic acid containing glycoproteins have been described in metacyclic *T. cruzi* by Schenkman et al. (1993).

Beecroft et al., 1993) as one major trypanosomal sialic acid acceptor (see below). The bloodstream stages of both, *T. brucei ssp.* and *T. congolense* (cultivated in mice or axenically in serum-containing medium) lack sialic acids as do the trans-sialidase-negative species *T. equiperdum* and *T. evansi*. Furthermore, neither *Leishmania*, *Leptomonas*, *Phytomonas*, *Crithidia* and *Herpetomonas*, nor *Giardia* and *Entamoeba* contain sialic acids. These results further confirm the assumption that trans-sialylation may be the only way in which the *Trypanosomatida* obtain sialic acids.

Characterization of procyclic T. congolense trans-sialidase activity

In supernatants from procyclic *T. congolense* cultures, a novel trans-sialidase activity was identified. Following three washing steps, no enzyme remained to be associated with the harvested trypanosomes. Specific trans-sialidase activity was 3.5 mU/mg protein.

In 100 mM Bis-Tris-buffer, the pH-optimum of *T. congolense* trans-sialidase was at pH 7.0. Still 70% of this maximum catalytic activity was found at pH 4.5 in 100 mM MES-buffer and at pH 8.5 in 100 mM TES-buffer, respectively. Activity optima at neutral pH-values have also been described for *T.b. brucei* and *T. cruzi* trans-sialidases (reviewed in Engstler and Schauer, 1993). The catalytic activity of the *T. congolense* enzyme showed a temperature optimum at 37°C in 100 mM Bis-Tris, pH 7.0, while at 15°C and 50°C, respectively, only 50% of the initial enzymic activity was measured.

After 5 h of incubation at 37°C, *T. congolense* trans-sialidase activity decreased to 50%. Storage at 4°C (>56 h) did not affect enzyme activity. Lyophilization and dissolution or 5 freeze-thaw cycles did not significantly alter the catalytic activity. In a range from pH 5.0–7.5, *T. congolense* trans-sialidase activity was stable, while after preincubations for 20 min at pH 4.0 and 8.5, respectively, only half of the initial enzyme activity could be measured.

The trans-sialidase activity from *T. congolense* is completely inhibited by the addition of 8 μM $HgCl_2$ or 14 μM p-hydroxymercuribenzoic acid. The enzyme activity shows neither a requirement for Ca^{2+} nor is it inhibited by 0.1–5 mM EDTA or EGTA. The addition of 30 mM Mg^{2+} or 3 mM Zn^{2+} did not affect catalytic activity, while incubations in the presence of 2.5 mM Fe^{2+}, 4.5 mM Cu^{2+} or 4 mM Ni^{2+}, respectively, decreased trans-sialidase activity to 50%.

Increasing concentrations of sodium chloride (0–1000 mM) reduce the enzyme activity. When *T. congolense* trans-sialidase was incubated in 100 mM Bis-Tris buffer, pH 7.0, containing 1 M NaCl, a decrease of enzymic activity to 40% was observed, whereas incubations with 1 M NaCl in 100 mM MES-buffer at pH 5.0 or in 100 mM TES-buffer at pH 8.5, yielded 67% and 20%, respectively, of the initial trans-sialidase activity. Thus, the influence of NaCl on *T. congolense* trans-sialidase activity is pH-dependent. Also other chlorides, e.g. KCl, affect *T. congolense* trans-sialidase activity, while other sodium salts, e.g. NaH_2PO_4, do not influence the catalytic activity (data not shown). A similar sensitivity towards chlorides has been described for *T.b. brucei* trans-sialidase (Engstler et al., 1993).

The sialidase inhibitor Neu5Ac2en (1 mM) reduces *T. congolense* trans-sialidase activity by 50%. Incubations of trans-sialidase in the presence of 2 mM or 10 mM Neu5Ac2en, respectively, yielded 65% and 100% loss of enzyme activity. The addition of the sialidase-inhibitor Siastatin B (5 mM), had no influence on enzymic activity. Furthermore, N-(4-nitrophenyl)oxamic acid did not affect the catalytic activity,

even at 10 mM concentration. The latter finding confirms the observation that N-acyl-anilines, which are known to be competitive inhibitors of e.g. *Vibrio cholerae* sialidase, generally might not act on trans-sialidases (Engstler et al., 1994). Both, Neu5Ac (10 mM) and CMP-Neu5Ac (5 mM), did not inhibit *T. congolense* trans-sialidase activity.

Substrate specificity

A number of sialoglycoconjugates have been tested as potential sialic acid donor compounds for *T. congolense* trans-sialidase (Table 3A). The best donor substrate found, was Neu5Ac-α(2–3)lactose. No difference in transfer rate was observed when the hydroxylated sialotrisaccharide Neu5Gc-α(2–3)lactose was used instead of Neu5Ac-α(2–3)lactose. Thus, for the first time, we report that N-glycoloyl-neuraminic acid can be trans-sialylated. Also *T. brucei* trans-sialidase does not distinguish between bound Neu5Ac and Neu5Gc. When compared to the above donor compounds, a more than 10-times slower transfer rate is obtained by using Neu5Ac-α(2–6)lactose. Sialic acids in α(2–8)linkage are trans-sialylated even 40-times slower than α(2–3)-bound sialic acids. Fetuin was a more efficient donor than the fluorigenic sialidase-substrate MU-Neu5Ac. Sialic acids on gangliosides or mucins did not serve as donor substrates. Furthermore, the trans-sialidase from *T. congolense* does not utilize CMP-Neu5Ac or free Neu5Ac as donor compounds. The major surface glycoprotein GARP, which recently has been described in procyclic *T. congolense* (Beecroft et al., 1993), is trans-sialylated by the action of *T. congolense* trans-sialidase. Interestingly, the *T. brucei* GARP-analogue protein, PARP/procyclin, is an equally good substrate also for the trans-sialidase from *T. congolense*.

Various compounds have been tested for their ability to present sialic acid acceptor sites for *T. congolense* trans-sialidase (Table 3B). In a standard assay, 2 mM MU-Neu5Ac were used as sialic acid donor and 200 000 dpm [^{14}C]lactose as the acceptor. The addition of potential competing acceptors to the reaction mixture resulted in a decrease of sialyl-[^{14}C]lactose formed. In parallel, the same experiments have been conducted using 2 mM sialyllactose as donor and 2 mM Gal-MU as the acceptor. Here, the transfer rate was measured as the relative decrease in fluorescence. Both assays led to almost identical results: *T. congolense* trans-sialidase transfers sialic acids exclusively onto terminal β-galactose residues, as in lactose or biantennary oligosaccharides. The enzyme does not accept α-linked galactose or any other saccharide tested as substrate. A re-transfer of Neu5Ac onto hydrolyzed MU-Neu5Ac is excluded. However, synthetic compounds, containing terminal β-galactose residues, such as 4-methylumbelliferyl-β-D-galactopyranoside (Gal-MU) or 4-nitrophenyl-β-D-galactopyranoside, respectively, are as good trans-sialylation acceptors as unmodified lactose. The E-selectin ligand Lewis^x is sialylated by *T. congolense* trans-sialidase activity to a minor degree. However, trans-sialylation seems to be the only enzymatic way to sialylate the pentasaccharide Lewis^x.

The desialylated GARP-surface protein from procyclic *T. congolense* accepts sialic acids in the trans-sialidase reaction. Thus, at least in vitro, one potential natural sialic acid acceptor molecule on the surface of *T. congolense* is identified. Instead of GARP also the desialylated *T. brucei*-glycoprotein PARP/procyclin can be used as sialic acid acceptor for the *T. congolense* enzyme. These findings underline the striking functional similarities between *T. brucei* and *T. congolense* trans-sialidase activities. GARP has been shown not to be simply a homologue to PARP, since the

TABLE 3

Substrate specificity of trans-sialidase from procyclic *Trypanosoma congolense*

A. Glycoconjugate	Relative transfer rate (%)
Neu5Ac-α(2–3)lactose	100
Neu5Gc-α(2–3)lactose	100
Fetuin	30
GARP (*T. congolense*)	24
PARP/Procyclin (*T. brucei*)	20
MU-Neu5Ac	18
Neu5Ac-α(2–6)lactose	8
Neu5Ac-α(2–8)Neu5Ac	2.5
Bovine brain ganglioside mixture	0
GD1a	0
Collocalia mucin	0
Bovine submandibular gland mucin	0
Neu5Ac	0
CMP-Neu5Ac	0
B. Compound	**Relative fluorescence (decrease in %)**
Lactose	94
4-Methylumbelliferyl-β-D-galactopyranoside[1]	90
Biantennary oligosaccharide[2]	85
4-Nitrophenyl-β-D-galactopyranoside	62
Asialofetuin[3]	65
IPTG	42
Asialo-GARP[3]	20
Asialo-PARP/Procyclin[3]	16
Methyl-β-D-galactopyranoside	16
Methyl-α-D-galactopyranoside	0
Lactose-BSA	9
Lewis^x[4]	15
Galactose	4
GalNAcα1–3[Fucα1–2]Gal	0
Mannose	0
Glucose	0
Methylumbelliferone[1]	0
Neu5Ac	0

A. Relative transfer rate of sialic acids from sialic acid containing compounds onto 2 mM lactose or 2 mM Gal-MU (for details see Materials and Methods). All potential substrates contained 1 mM of bound sialic acids.

B. Ability of some compounds to act as sialic acid acceptor for *T. congolense* trans-sialidase. Results are given as percent reduction in the synthesis of sialyl-Gal-MU as described in the text.

1 Given as percent reduction in the synthesis of sialyl[^{14}C]lactose.

2 Structure of biantennary oligosaccharide:

Galβ1–4GlcNAcβ1–2Manα1$_6$\
Manβ1–4GlcNAcβ1–4GlcNAc
Galβ1–4GlcNAcβ1–2Manα1^3/

3 The concentration of these compounds refers to the amount of sialic acids released by the action of *Vibrio cholerae* sialidase.

4 Structure of lewis^x: Galβ1–4[Fucα1–3]GlcNAcβ1–3Galβ1–4Glc.

primary structures of both proteins are different (Bayne et al., 1993). However, the two proteins share several features and are anchored to the outer parasite surface by similar unusual GPI-structures, which both may be trans-sialylated.

At least in procyclic *T. brucei*, the main function of trans-sialidase is to sialylate the GPI-anchor of PARP/procyclin. In this way a dense, charged glycocalyx is formed, which covers the entire trypanosomal membrane. The parasites are thought to become inaffectable by the attack of proteases, glycosidases, natural antibodies or complement from the fly's digestive system or from the ingested bloodmeal. Thus, the true function of both, PARP and GARP may lie within their GPI-anchors, which form an inpenetrable barrier after trans-sialylation.

It is striking that trans-sialylation is only found in those African trypanosomes which have to reside as dividing forms within the tsetse fly's midgut. Species which are transmitted mechanically or by other vectors seem to have no need for a protecting procyclic surface coat or reveal other protective mechanisms. This may be true for the *Leishmania*, which have to survive also within the gut of blood-sucking insects (and do not express trans-sialidase). The occurrence of trans-sialylation only in some trypanosomal species further promotes speculations on the evolutionary origin of this event. Recent studies on the evolution of nuclear ribosomal RNAs from kinetoplastid protozoa underline the antiquity of the group (Fernandes et al., 1993): The genera *Leishmania*, *Endotrypanum*, *Leptomonas* and *Crithidia* are closely related, while *T. cruzi* is more distantly related, and the *T. brucei*-group forms the deepest branch within the trypanosomatid protozoa. Since very recent results reveal, that also *Endotrypanum* does express trans-sialidase activity (Medina-Acosta et al., 1994), this enzyme now has been detected in all three parts of the tree. However, trans-sialylation is not a common feature among the trypanosomatids. The primary structures of *T. cruzi* trans-sialidases (reviewed in Cross and Takle, 1993) and a trans-sialidase-like protein from *T. rangeli* (Buschiazzo et al., 1993) exhibit considerable homology to bacterial sialidases (Roggentin et al., 1989). This may also be true for *T. brucei* trans-sialidase (M. Engstler, R. Schauer and P. Roggentin, unpublished data). Since it has been discussed that, at least in bacteria, sialidase genes may originate from higher eukaryotes by horizontal gene transfer (Roggentin et al., 1993), one may speculate whether this is also true for the trypanosomes. Trans-sialidase could have originally been acquired by the trypanosomatids from their hosts as a sialidase which was subsequently modified to act as trans-sialidase. With the generation of trans-sialidase, the ancient trypanosomes became capable of exploiting the phylogenetically young sialic acids for their survival strategies. The above speculations are supported by the occurrence of regular sialidases (without trans-sialidase activity) in *T. vivax* and *T. rangeli*.

It will be interesting to investigate, whether trans-sialylation has been developed more than one time during the evolution of parasitism (Engstler and Schauer, 1994). We are convinced that research on trans-sialidases will not only yield potent tools for glycotechnology (Ito and Paulson, 1993), but will also provide new insight into the biology and evolution of these ancient parasites.

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